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PRINCIPAL INVESTIGATOR: Kami Kim, MD

CONTRACTING ORGANIZATION: Albert Einstein College of Medicine
Bronx NY 10461

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Kami Kim, MD

E-Mail: kkim@aecom.yu.edu

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14. ABSTRACT: Because resistance to current antimalarials is widespread, new targets for malaria chemotherapy are needed to protect military personnel stationed in developing countries. Malaria parasites cannot make purines needed for RNA and DNA and must salvage purines from their host. The purine salvage pathway is unique in malaria parasites. We are investigating whether the specificity of these malaria enzymes can be exploited to develop specific treatments for malaria that will be effective but not toxic. While study of drug targets in vivo is critical for all infectious diseases, evaluation in an animal model is especially critical for evaluation of purine salvage as a drug target. We perform our studies in Plasmodium yoelii, a rodent malaria. We have developed GFP and GFP-luciferase reporter P. yoelii parasite lines. These reporter parasites can be visualized in mosquitoes as well as in infected mice. We have genetically disrupted purine salvage enzyme purine nucleoside phosphorylase (PNP) and have shown that these P.yoelii parasites are attenuated and confer protective immunity to subsequent challenge. Attempts to disrupt adenosine deaminase (ADA) have failed, suggesting it is an essential enzyme. We are testing the effects of malaria-specific PNP inhibitors on malaria infection in mice. We are also developing methods to test efficacy against exoerythrocytic malaria forms. We hope these experiments will lead to the development of new effective and nontoxic agents that can protect our military personnel from the lethal effects of malaria infection.

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Evaluation of Purine Salvage as a Chemotherapeutic Target in the *Plasmodium yoelii* Rodent Model
W81XWH-05-2-0025
Kami Kim, MD

I apologize for the delayed submission of this revised report. In the time between original submission March 1 and the request for a more detailed report in August, we have made substantial progress that I wished to clarify since the new results now invalidate some of the conclusions reported originally. We had thought we would not be able to accomplish task 2.

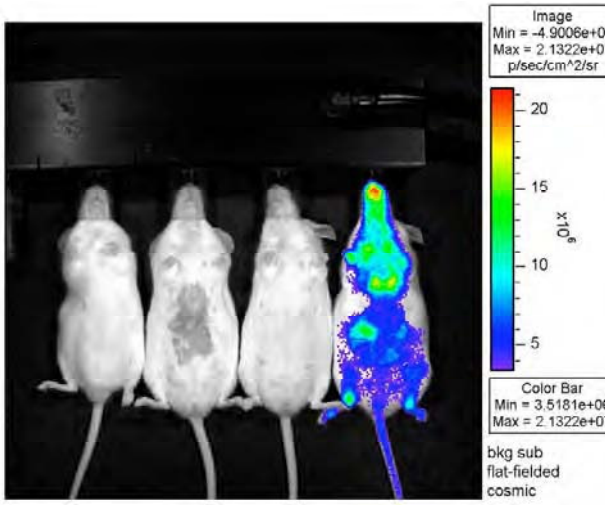
Introduction:

Because resistance to current antimalarials is widespread, new targets for malaria chemotherapy are needed to protect military personnel stationed in developing countries. Malaria parasites cannot make purines needed for RNA and DNA and must salvage purines from their host. Our preliminary studies reveal purine salvage is unique in malaria parasites. We would like to determine whether the unique activities of the malaria enzymes can be exploited to develop specific treatments for malaria that will be effective but not toxic. While study of drug targets in vivo is critical for all infectious diseases, evaluation in an animal model is especially critical for evaluation of purine salvage as a drug target. Malaria parasites are routinely maintained in the laboratory with high concentrations of purines, but levels of purines in mammalian blood are tightly regulated and 100-fold less than typical culture conditions. Therefore the efficacy of purine salvage inhibition and importance of purine salvage enzymes must be examined under physiological conditions that cannot be replicated during in vitro culture conditions. We plan to perform our studies in *Plasmodium yoelii,* a rodent malaria whose genome has been sequenced and for which there are techniques for genetic manipulation. Using this system we will genetically disrupt purine salvage genes and test their importance to the parasite. We will test the effects of malaria-specific purine salvage inhibitors on malaria infection in mice. These novel drugs will be tested in combination with other antimalarials and will also be evaluated for efficacy against exoerythrocytic malaria forms. We hope these experiments will lead to the development of new effective and nontoxic agents that can protect our military personnel from the lethal effects of malaria infection.

Task 1 PNP gene disruption in Plasmodium yoelii.

1. Make double cross-over constructs for PyPNP including one that replaces P. falciparum PNP for PyPNP. Months 1-3, year 1
2. Transfect, select and clone disruptants in mice. Months 4-12 year 1.
3. Analyze phenotype of disruptants and PfPNP replacements for mouse virulence, ability to complete the full life cycle, ability to transport and incorporate purines. Year 1 and year 2.

We have extended our work with *Plasmodium yoelii* transfection. We have made stable clones that express GFP (green fluorescent protein) under the EF1α promoter and stable clones that express the GFP/luc fusion protein (this is protein has both green fluorescence and luciferase



Uninfected WT A-IV C-IV
74% 30% 34% (parasitemia)

Figure 1
Pyoelii wild-type(WT); luc– clone (A-IV), luc+clone (C-IV) after injection of luciferin

reporter activity). These parasites clones are able to transit the complete life-cycle and retain reporter expression. Further we have shown, in collaboration with the group of Richard Novick at New York University, that the GFP/luc clones can be detected *in vivo* in infected mice after injection with luciferin, the substrate of luciferase (encoded by the *luc* reporter) (Figure 1). We plan to test examine *in vivo* infection and dissemination of these lines, after infection with sporozoites, with the eventual goal of using these lines for drug testing.

We have further analyzed single cross-over PNP disruptants (PypnpINT). By analyzing these disruptants immediately after cloning we have been able to characterize their phenotype. In parallel we analyzed a recombinant parasite clone transfected with a construct that integrated in the same region replacing the 3'UTR, but not disrupting *PNP* (PypnpWT). While the disruptants grow more slowly the 3'UTR control parasites grow normally (Figure 2). Only the disruptant is attenuated in mice, and unlike mice infected with the control strain, all mice infected with the disruptant survive (Figure 2). Survivors are resistant to subsequent lethal challenge with *P. yoelii.*

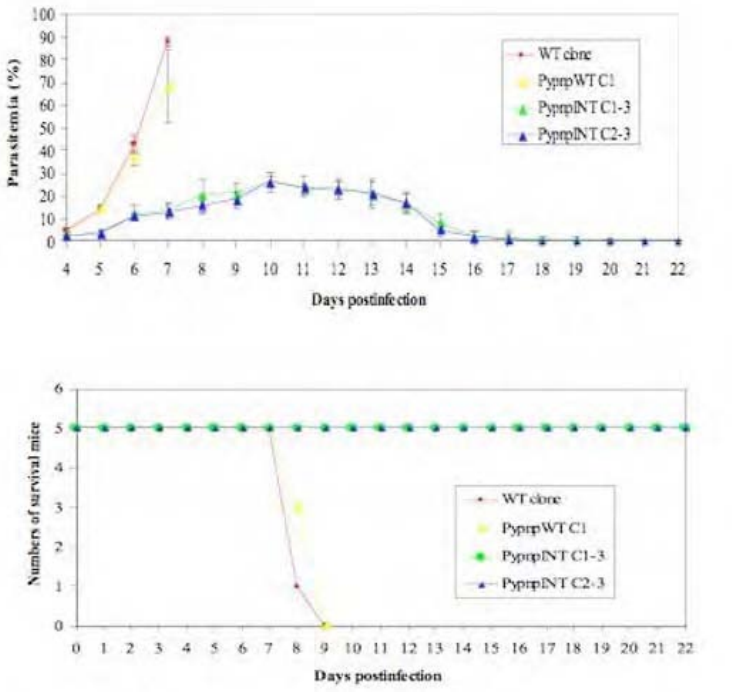


Figure 2: Parasitemias and survival of mice infected with wild-type, PNP disruptant (PypnpINT) or integrant with intact PNP (PypnpWT).

As reported in *P. berghei*, double crossover gene disruption in *P.yoelii* appears to be less efficient. After much effort we have successfully implemented double crossover double homologous recombination protocols. These constructs have been made using the PCR-based protocols of Ecker *et al*. We have been able to create double crossover gene deletions of *PyPNP*. All clones lacking PNP (*Δpypnp*) are attenuated in mice and mice are able to survive a lethal inoculum as for clones with gene disruption whereas clones with disruption in CSP (*Δpyscp*), the major

sporozoite antigen not required for erythrocytic growth, behave like wild-type malaria (data not shown). Mice infected with the *Δpypnp* clones are also protected from subsequent lethal challenge.

Since purines are essential for parasite development in all phases of the life cycle, in collaboration with the laboratory of Photini Sinnis, New York University School of Medicine, we tested the ability of the PNP KO clone to develop into the sexual stages.

TABLE 1 Parasite development in mosquitoes

Experiment	Parasite clone	No. of midgut oocysts per mosquito[a]	No. of salivary gland sporozoites per mosquito[b]	No. of infected/No. of mice biten by mosquitoes[c]/pre-patency[d]/genotype[e]
1	WT clone	47.7	11,875	2/2/d3/WT
	*Pypnp*WT C1	45.4	11,000	2/2/d3/*Pypnp*WT
	*Pypnp*INT C1-3	2.8	0	0/2/-/-
2	WT clone	42.8	7,750	2/2/d3/WT
	*Pypnp*WT C2	40.6	8,000	2/2/d3/PypnpWT
	*Pypnp*INT C1-4	1.5	0	1/2/d4/WT
3	WT clone	46.8	8,875	2/2/d3/WT
	Δ*Pycsp* C2-2	41.4	0	0/2/-/-
	Δ*Pypnp* C4-5	0	0	0/2/-/-
	Δ*Pypnp* C8	0	0	0/2/-/-

[a]Mean number of oocysts per midgut was determine by dissecting at least 20 mosquitoes at day 8.
[b]Mean number of sporozoites per salivary gland was determine by dissecting at least 20 mosquitoes at day 15.
[c]Group of 2 female Swiss Webster mice were infected with sporozoites by mosquito bite.
[d]Number of days after mosquito bite back until detectable blood stage parasitemia by microscopic blood smear examination.
[e]Genotype of blood stage parasites recovered after sporozoite infection by mosquitoes was analyzed by PCR using genomic DNA as template.

Our findings with the *Δpypnp* parasites are now being written up for submission for publication. We are also planning a methods paper delineating our improved protocols for genetic manipulation of *Plasmodium yoelii* parasites.

Task 2 ADA disruption in *Plasmodium yoelii*.

1. Make single and double cross-over disruption constructs. Months 10-12, year 1
2. Transfect, select and clone disruptants. Months 1-6, year 2.
3. Analyze phenotype of disruptants for mouse virulence, ability to complete the full life cycle, ability to transport and incorporate purines. Months 7-12 year 2. Continue year 3

We have made single cross-over disruption constructs for PyADA and double cross-over constructs. The ADA single cross-over construct has been transfected into *P. yoelii* numerous times. After selection we could detect PCR bands consistent with successful disruption of the ADA gene. The proportion of disruptants is consistently quite low and disappears with time. Despite optimization of transfection and selection conditions, we were not able to clone disruptants. In retrospect this was probably due to reversion and overgrowth of revertants.

After our successful development of double crossover gene deletion methodology (see aim 1), we were able to quickly delete the *PyADA* gene. A preliminary analysis of the phenotype shows similar results to the PNP deletions but comprehensive analysis is ongoing. Although there was a delay in implementing this aim, we are now on target to complete this aim.

Task 3 Test the effect of immucillins on the P. yoelii rodent malaria model.

1. Test whether the immucillins with best in vitro activity or best malaria-specific activity can cure mice with malaria. Year 1-2
2. Test whether effective immucillins are effective against transgenic *P. yoelii* that carry PfPNP. Year 3
3. Compare effects of malaria specific immucillins to immucillins that inhibit only mouse PNP or inhibit both PNPs in wild-type and parasites with disruption in purine salvage genes. Year 3

We have tested Immucillin-H, DADMe Immucillin-H, MT-Immucillin-H, and MT-coformycin against *P. yoelii* and *P.berghei.* Each of these inhibitors are able to kill cultured *P. falciparum* (Ting *et al,* and Madrid & Kim, unpublished). Unfortunately, none are able to cure mice. MT-ImmH, an inhibitor with 112-fold specificity for *P. falciparum* PNP, gave the best results with delay to death in some experiments. MT-coformycin is >20,000 more specific for PfADA over human ADA but was not efficacious.

TABLE 2 Kinetic constants of *Plasmodium* PNP

Substrate	*P. yoelii* PNP			*P.falciparum* PNP		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\ s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\ s^{-1}$)
Inosine	8.6 ± 1.5	3.45 ± 0.14	4.0×10^5	4.7 ± 0.9	1.1 ± 0.2	2.3×10^5
Methylthioinosine	5.0 ± 0.4	3.4 ± 0.06	6.8×10^5	10.8 ± 0.9	2.6 ± 0.8	2.4×10^5
Guanosine	8.9 ± 1.6	3.75 ± 0.18	4.2×10^5	9.4 ± 1.2	2.6 ± 0.5	2.8×10^5
2'-deoxyinosine	79 ± 10	1.20 ± 0.05	1.5×10^4	91 ± 35	0.89 ± 0.3	9.8×10^3

Because of these studies, we questioned whether the rodent malaria enzymes were as sensitive to the inhibitors as their *P. falciparum* orthologues. Therefore we cloned and fully characterized the rodent malaria PyPNP and PyADA (See alignments following this text). Recombinant PyPNP was compared to PfPNP. Its enzyme kinetics and inhibition profile are identical to that of PfPNP. PyPNP is similar to PfPNP and metabolizes both inosine and methylthioinosine. Thus the inability of immucillins to cure mice infected with *P. yoelii* is not likely due to differences in the target PNP enzymes.

In parallel we cloned PNP from other malaria species since a possible reason for failure of the immucillins could be the known differences in purine metabolism between rodents and primates. Therefore, studies may need to be performed in primates rather than rodents. For the immucillins currently in human trials, immucillins were less efficacious in mice than in humans.

Table 3: PNP Substrate Kinetics for PNP from different malarias

PNP Kinetics

	Inosine			MTI		
Species	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m
	μM	s^{-1}	M	μM	s^{-1}	M
P. knowlesi	6.9 +0.8	0.19	2.8E+04	5.2+0.6	0.034	6.6E+03
P. cynomolgi	6.2 +0.6	0.082	1.3E+04	8.6+0.8	0.047	5.5E+03
P. vivax	5.4+0.8	0.12	2.2E+04	12+1.5	0.061	5.3E+03
P. falciparum	3.9+0.5	0.14	3.7E+04	14+3	0.030	2.1E+03
P. berghei	7.9+0.9	0.76	9.5E+04	13+2	0.13	9.5E+03

Table 4: PNP Kinetics of Inhibition using Immucillins

PNP Inhibitor Kinetics

	ImmH (nM)		MT-ImmH (nM)	
Species	K_i	K_i^*	K_i	K_i^*
*H. sapiens**	3.3 ± 0.2	0.056 ± 0.015	300 ± 80	ND
P. knowlesi	2.6 ± 0.3	0.63 ± 0.09	23 ± 3	7.5 ± 1
P. cynomolgi	2.9 ± 0.3	0.58 ± 0.09	27 ± 3	6.9 ± 0.4
P. vivax	3.0 ± 0.3	0.49 ± 0.6	32 ± 5	7.2 ± 0.7
*P. falciparum***	2.6 ± 0.3	0.64 ± 0.08	23 ± 7	7.6 ± 0.3
P. berghei	1.2 ± 1.9	0.4 ± 0.07	30 ± 4	7.6 ± 1.1

*as published in (Lewandowicz et al, 2005)

**previously determined to be K_i= 0.860 nM for ImmH and K_i = 22 nM K_i^* = 2.7 nM for MT-ImmH by (Lewandowicz et al, 2005; Ting et al, 2005)

We have also cloned and characterized PyADA and PbADA. These enzymes show activity against both adenosine and methylthioadenosine. However, they are less efficiently inhibited by MT-coformycin (250pM for PfADA vs approximately 150nM for both PyADA and PbADA), although their susceptibility to coformycin is comparable (about 250pM). Thus there are differences in the ADAs of the various malaria species.

Table 5: ADA Substrate Kinetics

ADA Kinetics

	Adenosine			*MTA*		
Species	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m
	μM	s^{-1}	M	μM	s^{-1}	M
P. knowlesi	254 ± 36	6.8	2.7E+04	19 ± 3	0.012	6.5E+02
P. cynomolgi	86.5 ± 9.4	5.3	6.2E+04	8 ± 5	0.028	1.4E+03
P. vivax	59.5 ± 5.9	1.8	3.1E+04	9.5 ± 0.8	0.13	1.4E+04
P. falciparum	25.1 ± 3.6	1.4	5.6E+04	110 ± 20	2.6	2.5E+04
P. berghei	56.8 ± 2.1	4.7	8.2E+04	4.4 ± 0.6	0.35	7.9E+04

Table 6: ADA Kinetics of Inhibition

ADA Inhibitor Kinetics

	Coformycin (nM)		MT-Coformycin (nM)	
Species	K_i	K_i^*	$\mathbf{K_i}$	$\mathbf{K_i^*}$
*H. sapiens**	2.9 ± 0.5	0.590 ± 0.05	> 10,000	ND
*B. Taurus***	8.8 ± 0.7	0.530 ± 0.06	> 10,000	ND
P. knowlesi	3.4 ± 0.7	0.639 ± 0.04	477 ± 69	ND
P. cynomolgi	7 ± 2	0.410 ± 0.04	295 ± 28	ND
P. vivax	7.4 ± 0.8	0.710 ± 0.09	200 ± 50	ND
*P. falciparum****	14 ± 3	0.260 ± 0.03	3.2 ± 0.55	0.25 ± 0.050
P. berghei	2.3 ± 0.4	0.152 ± 0.01	136 ± 16	ND

Previously reported values for these enzymes are (Tyler et al, 2007):
Coformycin: Ki = 1.1 nM, Ki = 0.06 nM / MT Coformycin: >10,000 nM
**Coformycin: Ki = 13.9 nM, Ki* = 0.11 nM / MT Coformycin: >10,000 nM
***Coformycin: Ki = 0.68 nM, Ki* = 0.08 nM / MT-Coformycin: Ki = 2.66 nM Ki* = 0.43 nM
ND = not detectible

Table II: Kinetic constants for mutant and wildtype PNPs from *P. falciparum* and *T. gondii*

PNP	Inosine Km μM	Inosine kcat s^{-1}	Inosine kcat/Km $M^{-1}s^{-1}$	5'-Methylthioinosine Km μM	5'-Methylthioinosine kcat s^{-1}	5'-Methylthioinosine kcat/Km $M^{-1}s^{-1}$
PfPNP	11.3 ± 5	1.72 ± 0.68	**$1.56x10^5$**	8.75 ± 0.2	0.83 ± 0.03	**$9.56x10^4$**
PfPNP[a]	4.7 ± 0.3	1.7 ± 0.3	$3.6x10^5$	16 ± 3	1.5 ± 0.2	$9.6x10^4$
TgPNP[b]	13.1 ± 1.2	2.60 ± 0.02	$1.98x10^5$	31.9 ± 2.7	0.027	$8.46x10^2$
Val66Ile	15.6 ± 4	2.04 ± 0.23	**$1.38x10^5$**	12.2 ± 5.2	1.15 ± 0.74	**$1.02x10^5$**
Val73Ile	13.5 ± 2.2	1.88 ± 0.69	**$1.37x10^5$**	11.4 ± 7	0.81 ± 0.03	**$1.22x10^5$**
Tyr160Phe	2.5 ± 1.2	0.21 ± 0.02	**$1.13x10^5$**	43.9 ± 4.7	0.30 ± 0.04	**$7.09x10^3$**
Val66Ile/Val73Ile	27.9 ± 11	2.81 ± 0.37	**$1.09x10^5$**	14.7 ± 5	0.91 ± 0.2	**$7.48x10^4$**
Val66Ile/Tyr160Phe	6.17 ± 3	0.70 ± 0.10	**$1.30x10^5$**	10.5 ± 1.5	0.05 ± 0.002	**$4.86x10^3$**
Val73Ile/Tyr160Phe	3.62 ± 1.3	0.59 ± 0.13	**$1.70x10^5$**	60.3 ± 32	0.28 ± 0.04	**$5.25x10^3$**
V66I/V73I/Y160F	3.96 ± 1.8	0.36 ± 0.23	**$8.94x10^4$**	17.8 ± 6	0.007	**$5.12x10^2$**

Values reported in Shi, *et al* (2004)[a], and Chaudhary, *et al* (2006)[b]

Now that we have resolved some of the technical difficulties, we have begun to create constructs to replace PyPNP with PfPNP (#2 in this task). Although these enzymes do not differ in kinetic properties, we have now mapped the key residues responsible for the methylthiospecificity of PfPNP (see table above) by taking advantage of the finding that TgPNP is homologous to PfPNP but does not have activity against methylthiopurines. By comparing differences in the amino acid sequences and examining the crystal structure of PfPNP (Shi *et al*) we were able to determine that mutations in 3 key residues: V66I, V73I, Y160F were needed loss of MTI activity. We will replace PyPNP with PfPNP with wild-type activity as well as with only activity against inosine to test the importance of this unique substrate specificity.

Task 4 Test immucillins in combination with antimalarials

1. Establish dose response of agents (DMFO, deoxycoformycin, atovoquone, mycophenolic acid) against *P. yoelii.* Years 2-3
2. Test additive and synergistic effects of agents (particularly polyamine and ADA inhibitors) with best immucillin(s).Year 3-4
3. Test best drug combination for "causal" prophylaxis and cure after sporozoite challenge using *P. yoelii* sporozoites. Year 4

Dose response studies for agents to be used in combination were initiated. Because the immucillins were not effective in the rodent models we shifted our focus to verifying that recombinant enzyme targets were susceptible (see above). Previous studies reveal that the immucillins are bioavailable in mice. It is possible that the differences in purine metabolism between mice and humans may play a factor in our results with immucillins.

We have screened a panel of polyamine analogs from Cellgate for efficacy against *P. falciparum.* Some had activity at high concentrations and we tested the most promising agents in a rodent model of malaria. Unfortunately, none of these inhibitors had efficacy in the mouse model.

A major area that needs further investigation is the liver stages of malaria. An initial pilot experiment showed that immucillins did not protect mice from malaria after challenge with sporozoites (in collaboration with Dr. Sinnis). Understanding and targeting these stages has major impact upon military objectives as agents that target the liver stages would be effective prophylactic agents. We have therefore initiated a parallel effort to develop tools to better evaluate the efficacy of agents against the liver stages. We have several cell lines including HepG2-CD81 cells, Hepa 1-6 cells, and primary hepatocytes that we have tested for invasion by sporozoites and exoerythrocytic form (EEF) development. As described by others, *P.yoelii* sporozoites do not invade or develop as well as *P.berghei,* but *P.yoelii* is felt to better reflect the biology of *P.falciparum,* the human malaria.

We plan to test the recombinant GFP and GFP/luc lines we have developed. These reporter parasites will be used to develop more robust assays for testing antimalarials against the liver stages. Once the assay is developed we will test immucillins as well as other agents. Since all developmental stages require purines, purine salvage inhibitors could be potential prophylactic agents. These assays will allow us to determine is liver stages are sensitive to the inhibitors. The lack of efficacy in rodent models could be due to , poor pharmacokinetics or altered purine pathways in the mouse in response to malaria infection leading to partial rescue of the parasites due to unanticipated purines able to bypass the inhibitor block.

Key Research Accomplishments:

1. Stable luciferase reporter line able to complete entire malaria life cycle.
2. Development of PCR-based methodology to rapidly knock-out genes by double homologous gene replacement in *P. yoelii.*
3. Disruption of PyPNP and illustration that these disruptants are attenuated and unable to form oocysts or sporozoites
4. Illustration that attenuated PyPNP are protective against subsequent lethal challenge.
5. Disruption of PyADA—full characterization ongoing.
6. Characterization and inhibition profile of PNP enzymes from rodent and primate malarias.
7. Characterization of enzyme kinetics of ADA enzymes from rodent and primate malarias.

Reportable Outcomes:

Stable *P.yoelii* GFP-luciferase reporter parasite
Stable *P.yoelii* clones with knockout of *pnp, ada* and *csp.*

Conclusions:

Transfection studies are consistent with the hypothesis that PNP is important to viability of malaria. Parasites lacking PNP are attenuated in blood stages, and have dramatically reduced development of oocysts. In addition, the attenuated blood stage parasites are protective against subsequent lethal challenge with *P. yoelii.* Attempts to disrupt ADA have finally been successful using a new technique for PCR mediated double homologous recombination, and preliminary

studies suggest these parasites are also attenuated. The efficacy of the immucillins against rodent malarias is less than expected, and we are investigating potential reasons for this. In parallel we have developed reporter parasites that are able to complete the entire life cycle and are likely to be useful for development of *in vivo* and *in vitro* protocols for drug testing.

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Appendices:

Appendix Figure 1:

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CLUSTAL W (1.83) multiple sequence alignment


PvivaxPNP           MEGEMQRHIKLTKAQTTPVVLVVGDPGRVDKVKVLCDSYVDLAYNREYKSVECTYKGQKF 60
PknowelsiPNP        MEEEMQRHIKLTPSQTTPVVLVVGDPGRVDKVKMLCDSYVDLAYNREYKSVECTYKGQKF 60
PcynomolgiPNP       MEGEMQRHIKLTKAQTTPVVLVVGDPGRVDKVKVLCDSYVDLAYNREYKSVECTYKGQKF 60
PyoeliiPNP          MD-EEQRHIKLSKKHATPVVLVVGDPGRVDKIKVLCDSYVDLACNREYKSVECHYKGQKF 59
Pberghei            MD-EEQRHIKLSKKHATPVVLVVGDPGRVDKIKVLCDSYVDLAYNREYKSVECHYKGQKF 59
PfalciparumPNP      MD-NLLRHLKISKEQITPVVLVVGDPGRVDKIKVVCDSYVDLAYNREYKSVECHYKGQKF 59

PvivaxPNP           LCVSHGVGSAGCAICFEELMNNGAKVIIRAGSCGSLQPTQMKRGDICICNAAVREDRVSH 120
PknowelsiPNP        LCVSHGVGSAGCAICFEELMNNGAKVIIRAGSCGSLQPTQMKRGDICICNAAVREDRVSH 120
PcynomolgiPNP       LCVSHGVGSAGCAICFEELMNNGAKVIIRAGSCGSLQPDEIKRGDICVCNAAVREDRVSH 120
PyoeliiPNP          LCVSHGVGSAGCAICFEELINIGAKVIIRAGSCGSLQPESIKRGDLCVCNAAVREDRVTH 119
Pberghei            LCVSHGVGSSGCAICFEELINIGAKVIIRAGSCGSLQPESIKRGDLCVCNAAVREDRVTH 119
PfalciparumPNP      LCVSHGVGSAGCAVCFEELCQNGAKVIIRAGSCGSLQPDLIKRGDICICNAAVREDRVSH 119

PvivaxPNP           LMIYSDFPAVADYEVYATLNQVAEELKVPVFNGISLSSDMYYPHKIIPTRLEDYSKANVA 180
PknowelsiPNP        LMIYSDFPAVADFEVYDTLNKVAQELEVPVFNGISLSSDLYYPHKIIPTRLEDYSKANVA 180
PcynomolgiPNP       LMIHSDFPAVADFEVYDTLNKVAQELKVPVFNGISLSSDMYYPHKIIPTRLEDYSKANVA 180
PyoeliiPNP          MMIHSDFPAVADYEVYSTLLKCAEELNVKVHNGISLSSDLYYPHSIIPTRLLDYSKANVA 179
Pberghei            MMIHSDFPAVADYEVYSTLLKCAEELNVKVHNGISLSSDLYYPHSIIPTRLLDYSKANVA 179
PfalciparumPNP      LLIHGDFPAVGDFDVYDTLNKCAQELNVPVFNGISVSSDMYYPNKIIPSRLEDYSKANAA 179

PvivaxPNP           VVEMEVATLMVMGTLRKVKTGGIFIVDGCPLKWDEGDFDNNLVPERLENMIKISLETCAR 240
PknowelsiPNP        VVEMEVATLMVMGTLRKVKTGGIFIVDGCPLKWDEGDFDNNLVPEKLENMIKISLETCAR 240
PcynomolgiPNP       VVEMEVATLMVMGTLRKVKTGGIFIVDGCPFKWDEGDFDNNLVPERLENMIKISLETCAR 240
PyoeliiPNP          VVEMELSTLMVMGTLKKVKTGGIFIVDGCPLKWDEGDFDNVLAADRLENMIKISLEACAR 239
Pberghei            VVEMELSTLMVMGTLKKVKTGGIFIVDGCPLKWDEGDFDNVLAADRLENMIKISLEACAK 239
PfalciparumPNP      VVEMELATLMVIGTLRKVKTGGILIVDGCPFKWDEGDFDNNLVPHQLENMIKIALGACAK 239

PvivaxPNP           LAKKY- 245
PknowelsiPNP        LAKKY- 245
PcynomolgiPNP       LAKKY- 245
PyoeliiPNP          LSEKY- 244
Pberghei            LSKKY- 244
PfalciparumPNP      LATKYA 245
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Amino acid sequence alignment for purine nucleoside phosphorylase (PNP) cloned from various species of malaria.

Appendix Figure 2:

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CLUSTAL W (1.83) multiple sequence alignment


PvivaxADA           -----MNILQEPIDFLKKEELKNIDLSQMSKKERYKIWKRIPKCELHCHLDLCFSADFFV 55
PcynomolgiADA       -----MNILQEPIDFLKKDEIKNIDLSQMSKKERYKIWKRIPKCELHCHLDLCFSADFFL 55
PknowlseiADA        -----MNILQEPIDFLKKDELKNIDLSQMDKKERYKIWKRIPKCELHCHLDLCFSADFFL 55
PfalciparumADA      MNCKNMDTSYEIINYLTKDELD-IDLSCMDKKERYKIWKRLPKCELHCHLDVCFSVDFFL 59
PbergheiADA         -----MEIPNEEIKFLKKEDIKNINLNGMNKKERYEIWKKIPKVELHCHLDLTFSGKFFL 55
PyoeliiADA          ----MMEIPTEEIKFLKKEDVQNIDLNGMSKKERYEIWRRIPKVELHCHLDLTFSAEFFL 56

PvivaxADA           SCIRKYNLQPNLSDEEVLDYYLFAKGGKSLGEFVEKAIKVADIFHDYEVIEDLAKHAVFN 115
PcynomolgiADA       SCIRKYNLQPNLSDEEVLDYYLFAKGGKSLGEFVEKAIRVADIFHDYEVIEDLAKHAVFN 115
PknowlseiADA        SCVRKYNLQPNLSDEEVLDYYLFAKGGKSLGEFVEKAIRVADIFQDYEMIEDLAKHAVFN 115
PfalciparumADA      NVIRKYNIQPNMSDEEIIDYYLFSKPGKSLDEFVEKALRLTDIYIDYTVVEDLAKHAVFN 119
PbergheiADA         KWVRKYNLQPNMTDDQVLDHYLFTKEGKSLAEFIRKAISVSDIYRDYDILEDLAKWAVIE 115
PyoeliiADA          KWARKYNLQPNMSDDEILDHYLFTKEGKSLAEFIRKAISVSDLYRDYDFIEDLAKWAVIE 116

PvivaxADA           KYKEGVVLMEFRYSPTFVAFKYNLDIELIHQAIVKGIKEVVELLDHKIHVALMCIGDTGH 175
PcynomolgiADA       KYKEGVVLMEFRYSPTFVAFKYKLDIELIHQAIVKGIKEVVELLDHKIHVALMCIGDTGH 175
PknowlseiADA        KYKEGVVLMEFRYSPTFVAFKHNLDIELIHQAIVKGIKEVVELLDHKIDVTLLCIGDTGH 175
PfalciparumADA      KYKEGVVLMEFRYSPSFMSFKHNLDKDLIHEAIVKGLNEAVALLEYKIQVGLLCTGDGGL 179
PbergheiADA         KYKEGVVLMEFRYSPTFVSSSHGLDIELIHKAFVKGIKNATEMLNNKIYVALICISDTGH 175
PyoeliiADA          KYKEGVVLMEFRYSPTFVSSSYGLDVELIHKAFIKGIKNATELLNNKIHVALICISDTGH 176

PvivaxADA           EAANIKASADFCLKHKADFVGFDHGGHEVDLKEYKEIFDYVRESGVPLSVHAGEDVTLPN 235
PcynomolgiADA       EAANIKASADFCLKHRADFVGFDHGGHEVDLKQYKEIFDYVRESGIPLSVHAGEDVTLPN 235
PknowlseiADA        RAADIKASADFCLKHKADFVGFDHGGHEVDLKPYKEIFDYVKEGGMHLTVHAGEDVTLPN 235
PfalciparumADA      SHERMKEAAEFCIKHKKDFVGYDHAGHEVDLKPFKDIFDNIREEGISLSVHAGEDVSIPN 239
PbergheiADA         SAASIKHSGDFAIKHKHDFVGFDHGGREIDLKDHKDVYHSVRNHGLHLTVHAGEDATLPN 235
PyoeliiADA          AAASIKHSGDFAIKHKHDFVGFDHGGREIDLKDHKDVYHSVRDHGLHLTVHAGEDATLPN 236

PvivaxADA           LNTLYSAIQVLKVERIGHGIRVAESQELIDMVKEKNILLEVCPISNVLLKNAKSMDTHPI 295
PcynomolgiADA       LNTLYSAIQVLKVERIGHGIRVSESQELIDMVKEKNILLEVCPISNVLLKNAKSMDTHPI 295
PknowlseiADA        LNTLYSAIQVLKVERIGHGIRVSESQELIDMVKENNILLEVCPISNVLLKNAKSFDTHPI 295
PfalciparumADA      LNSLYTAINLLHVKRIGHGIRVSESQELIDLVKEKDILLEVCPISNVLLNNVKSMDTHPI 299
PbergheiADA         LNTLYTAINILNVERIGHGIRVSESEELIELVKKNNILLEVCPISNLLLNNVKSMDTHPI 295
PyoeliiADA          LNTLYTAINILNVERIGHGIRVSESDELIELVKKKDILLEVCPISNLLLNNVKSMDTHPI 296

PvivaxADA           RQLYDAGVKVSVNSDDPGMFLTNINDDYEELYTHLNFTLEDFMKMNEWALEKSFMDSNIK 355
PcynomolgiADA       RQLYDAGVKVSVNSDDPGMFLTNINDDYEELYTHLNFTLEDFMKMNEWALEKSFMDSNIK 355
PknowlseiADA        RKLYDAGVKVSVSSDDPGMFLTNINDDYEKLYTHLHFTLEDFMKMNEWALEKSFIGCDIK 355
PfalciparumADA      RMLYDAGVKVSVNSDDPGMFLTNITDNYEELYTHLNFTLADFMKMNLWAVQKSFVDPDIK 359
PbergheiADA         RKLFDAGVKVSVNSDDPGMFLTDINDNYEKLYIHLNFTLEEFMTMNNWALEKSFVNDDIK 355
PyoeliiADA          RKLYDAGVKVSVNSDDPGMFLSNINDNYEKLYIHLNFTLEEFMIMNNWAFEKSFVSDDVK 356

PvivaxADA           DKIKNLYF 363
PcynomolgiADA       DKIKNLYF 363
PknowlseiADA        EKIKKLYF 363
PfalciparumADA      NKIISKYF 367
PbergheiADA         SKLKTMYF 363
PyoeliiADA          SELKALYF 364
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Amino acid sequence alignment for adenosine deaminase (ADA) cloned from various species of malaria.